Behavioral and Electrophysiological Experiments Suggest That the Antennular Outer Flagellum Is the Site of Pheromone Reception in the Male Helmet Crab *Telmessus cheiragonus*

MICHIYA KAMIO[1,*], MAKOTO ARAKI[2], TOSHIKI NAGAYAMA[2], SHIGEKI MATSUNAGA[1], AND NOBUHIRO FUSETANI[1]

[1] *Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku Tokyo 113-8657, Japan; and* [2] *Hokkaido University, Division of Biological Science, Graduate School of Science, Sapporo, Hokkaido 060, Japan*

Abstract. Sexually competent females of *Telmessus cheiragonus* (helmet crab) release two pheromones that elicit grasping and copulation behaviors in males (Kamio *et al.*, 2000, 2002, 2003). Our study aimed to use behavioral and electrophysiological techniques to identify the site of reception of these sex pheromones. In behavioral experiments, either the inner or the outer flagella of the antennules were ablated bilaterally from male crabs, and responses of male crabs to female odor were examined. When the inner flagella were surgically ablated, the sexual response (*i.e.*, grasping and copulation behavior) of male crabs was not significantly changed relative to control animals that had their second antennae ablated. In contrast, the sexual response was significantly reduced when the outer flagella of the antennules were ablated, suggesting that the outer flagellum is the receptor organ that detects the sex pheromones. In electrophysiological experiments, urine, which in females contains the pheromone that elicits grasping behavior by males but does not contain the pheromone eliciting copulation, whose release site is not known, was tested. Female and male urine as well as shrimp extract evoked phasic responses of chemosensory afferents innervating aesthetasc sensilla on the outer flagellum of male crabs. The response of the afferents had significantly higher magnitude and lower threshold when female urine was applied. Thus,

behavioral and electrophysiological observations suggest that in male helmet crabs, the outer flagellum of the antennule is the chemosensory organ that detects female sex pheromone.

Introduction

Sex pheromones are crucial chemosensory signals that trigger and modulate reproductive behaviors in conspecifics. In many animals, pheromones are detected by specialized receptor organs. For example, many vertebrates detect pheromones through their vomeronasal systems (Halpern and Martínez-Marcós, 2003), whereas insects have sensilla on their antennae dedicated to sensing pheromones (Hecker and Butenandt, 1984). Identification of receptor organs and electrophysiological analyses of chemoreception are powerful methods for isolating and identifying pheromone compounds (Bjostad, 1998; Li *et al.*, 2002).

In decapod crustaceans, chemoreceptors occur in high densities at multiple loci on the body and appendages. The antennules (first antennae), the pereiopod dactyls, and the mouthparts are the primary chemoreceptor organs (Ache, 1982). Among these organs, the antennules so far have been reported as pheromone receptors in certain crabs, including *Portunus sanguinolentus* (Christofferson, 1972), *Callinectes sapidus* (Gleeson, 1980), *Carcinus maenus* (Bamber and Naylor, 1996), and *Chionoecetes opilio* (Bouchard *et al.*, 1996). The antennules are biramous, with an inner and an outer flagellum. (The inner and outer flagella of crabs are equivalent to the medial and lateral flagella, respectively, of some other decapod crustaceans.) One type of sensillum

located exclusively on the ventral side of the outer flagella is the aesthetasc. Ablation experiments suggested that pheromones are detected by the outer flagella (Christofferson, 1972), specifically the aesthetascs (Gleeson, 1980). In the crayfish *Procambarus clarkii*, both inner and outer flagella are involved in mating behavior (Dunham and Oh, 1992), but the animals can still mate without their antennules (Corotto *et al.*, 1999). Ablation of antennules also affects mating behavior of female American lobsters, *Homarus americanus* (Cowan, 1991). However, only a small body of neurological evidence supporting these behavioral observations has been obtained. No electrophysiological analyses were performed to demonstrate chemosensory responses of the afferents of the outer flagellum to sex pheromones.

Mating behaviors of male helmet crabs, *Telmessus cheiragonus* (Tilesius, 1815) (Decapoda: Brachyura: Cheiragonidae) are elicited by pheromones that are released from females (Kamio *et al.*, 2003). One pheromone, present in the urine, elicits precopulatory guarding (Kamio *et al.*, 2000), whereas another pheromone, from unknown sources of the postmolt female, subsequently elicits copulation behavior. These findings are based on a series of observations of the behavior of males towards artificial sponges that contain female odors. Sponges that contained aquarium water conditioned by the presence of postmolt females elicited grasping and then copulation behavior, while sponges that contained aquarium water of premolt females elicited only grasping behavior. Moreover, urine from both pre- and postmolt females elicited grasping behavior but did not elicit copulation behavior (Kamio *et al.*, 2002).

In the present paper, the location of pheromone receptors was assessed by behavioral experiments using males that underwent surgical ablation of the inner or outer flagella. Mating behavior of male crabs was eliminated after ablation of the outer flagella. Chemosensory responses of the outer flagella afferents were examined using electrophysiological recordings that integrate multiunit responses of the chemosensory afferents. These recordings showed significantly stronger responses of the afferents to female urine than to male urine. Thus, the behavioral experiments suggest that the outer flagellum possesses pheromone receptors, and the electrophysiological experiments support this idea.

Materials and Methods

Animals

Pre-copula pairs of *Telmessus cheiragonus* (helmet crabs) were collected during the mating season between May and June 2001 and 2002 from pier walls in Usujiri, Hokkaido, Japan (N 41° 57′, E 140° 58′). Males were separated from their partner females and were maintained at 10.5 ± 1 °C in an aquarium with a recirculating seawater system under natural photoperiod. Females were housed in a separate flow-through seawater system until used at ambient temper-

ature. Behavioral experiments were conducted at the Usujiri Marine Biological Station, and electrophysiological experiments were conducted at Hokkaido University, Graduate School of Science, Sapporo. Males were fed shrimp, but females were not fed because premolt females and newly postmolt females do not eat.

Behavioral experiments

Behavioral experiments were conducted to test the effects of bilateral ablation of inner or outer flagella on the responsiveness of male crabs towards female pheromones. Three types of ablated males were prepared: (1) *inner flagellum ablated*, in which the inner flagella of the antennules (first antennae) of males were ablated at their base; (2) *outer flagellum ablated*, in which the outer flagella of the antennules of males were ablated at their base (see Fig. 1); and (3) *second antenna ablated*, in which second antennae were removed as a control for the trauma of surgery. All surgeries were carried out 3 days before the experiments, because no negative effects of the surgery alone on feeding behavior were observed at this time. Behavioral experiments were performed blind, with the observer being unaware of the type of ablation received by the crab under study.

The responses of the three types of ablated males toward female pheromone were observed using artificial sponges as female dummies (Kamio *et al.*, 2002). The sponges were washed with seawater before use. Male crabs were individually maintained at 10 °C in aquaria with a recirculating seawater system. For behavioral testing, a crab was transferred to a test aquarium with still water (31.5 × 18.5 × 24.4 cm), acclimated at 15 °C for 4 h, and then observed under artificial red light. A sponge (2.5 × 2.5 × 4 cm), which had been incubated for 20 h in a 5-liter aquarium containing 10 postmolt females that had molted 2–3 days before, was placed, using forceps, in front of a male until it contacted his chelae. Then the behavior of the male crab was observed for 1 h. Two particular behaviors were recorded as unmistakable and reliable criteria for sexual reaction: (1) *grasping*, a behavior in which the male grasps and fumbles with the sponge, without eating behavior, and places the sponge into the precopulatory guard position; and (2) *copulation*, a behavior in which the male first grasps the sponge, then opens his abdomen to expose the sexual appendages, and then moves the sponge onto the gonopods for insertion.

Electrophysiology

Electrophysiological recordings were performed to test the responses of chemoreceptor neurons in the outer flagellum of males to female odors. Multiunit spike responses of these neurons were recorded, using oil-hook extracellular electrodes, from nerve bundles innervating the antennule (Fig. 1). The excised antennule was placed in a recording
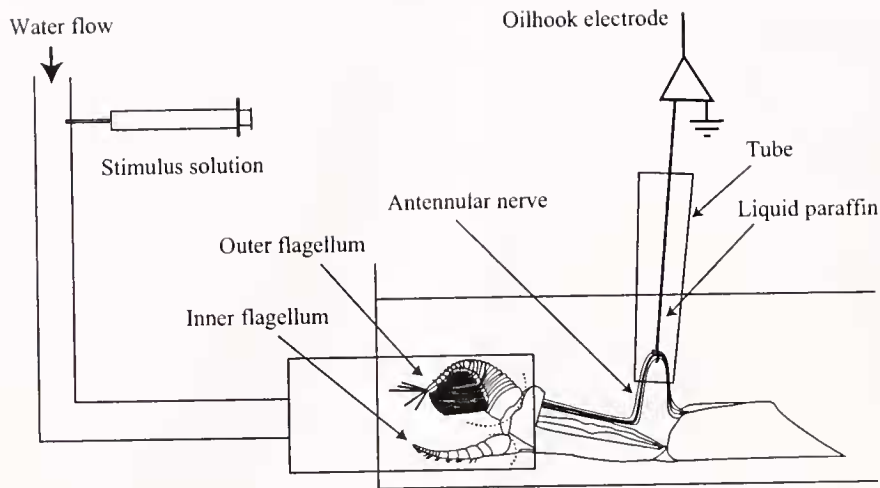
Figure 1. Diagram of the olfactometer used in recording from the antennular nerve of male specimens of *Telmessus cheiragonus*, and ablation points in behavioral experiments. Drawing of lateral view of left antennule. Ablation points of outer and inner flagella are indicated by dashed lines.

chamber. The dorsal cuticle of the distal podomere of the antennule was removed to expose the nerve bundles of the antennule. The nerve bundles were lifted up at the joint of the antennule and placed on a silver hook electrode that could be drawn into a capillary filled with liquid paraffin (Wilkens and Wolf, 1974). A carrier stream of cooled artificial seawater (ASW) (12–16 °C) continuously flowed in a tube past the tip of the antennule. After the inner flagellum had been ablated at the base, the outer flagellum was placed inside the tube.

Electrical signals were amplified with a bioelectric amplifier (NIHON KOUDEN MEG-2100), recorded digitally, and analyzed off-line using a microcomputer with hardware/software utilities (Powerlab, AD Instrumental). The multiunit response of the sensory afferents was integrated with a time constant of 2 s to obtain a measure of the magnitude of response (Caprio and Robinson, 1989; Sveinsson and Hara, 2000). Test stimulus solutions were kept cool during the experiment. Ten microliters of each stimulus solution was injected into the tube.

A food odor—shrimp extract—was prepared by homogenizing a small piece of fresh shrimp muscle (1.5 ml) in 30 ml of ASW. This mixture was centrifuged, which yielded a supernatant that was diluted 10 times and constituted the shrimp extract. Instead of aquarium water, the urine of female and male crabs was used for this experiment because it represents a more highly concentrated and less contaminated source of pheromone. Female urine, which contains pheromone that elicits grasping behavior by males (Kamio et al., 2002), was collected and pooled from 426 postmolt female crabs. Urine was collected from the antennal gland opening by lifting up the operculum covering the gland opening and using a peristaltic pump to collect the urine as it streamed out of the opening (Kamio et al., 2000). Male

urine was collected from 20 individuals. Urine was tested at a 1/10 dilution.

Chemosensory responses of male crabs to ASW, shrimp extract, male urine, and female urine were recorded sequentially. Subsequently, the outer flagellum was removed at a point described in Figure 2 and used in a test of shrimp extract to determine whether the chemosensory response recorded before was actually derived from the outer flagellum ($n = 11$). The magnitude of the responses was normalized to the response obtained with shrimp extract. In a different experiment to compare the sensitivity of chemoreceptor neurons of the male crab to female and male urines, a recording and data analysis strategy similar to that of Sveinsson and Hara (1990) was used. Solutions were always tested in order of ascending concentration with 3-min intervals. L-Serine (Wako Chemical Co.) at 10^{-3} M was used as a standard reference stimulus (STD), because the response to L-serine was phasic and did not seem to affect any subsequent responses. The STD was tested at the beginning and end of each test period. If the magnitude of STD changed more than 30% during a test period, the results were excluded from the data analysis. The responses of chemosensory neurons were normalized as relative magnitudes of STD for each test period. Differences in sensitivity of male crabs to both male and female urines at each concentration and to amino acids were statistically tested by Wilcoxon matched pairs test ($n = 6$).

To compare the concentrations of common stimulants for the aesthetasc, amino acids and other nitrogenous compounds (Spencer, 1986; Derby and Atema, 1988) were analyzed. To determine the contents of free amino acid in the pooled urine of intermolt males and the pooled urine of postmolt females, 2-ml aliquots of the urines were lyophilized, dissolved in 100 μl of distilled water, and centrifuged.

To remove proteins, a 200-μl portion of a 0.5% aqueous 5-sulfosalicylic acid solution was added to the supernatant. A 20-μl aliquot of this solution was injected into a model L 8500A Hitachi amino acid analyzer. This method allowed determination of concentrations of 20 common amino acids, as well as phosphoserine (P-Ser), taurine (Tau), urea, citrulline (Cit), sarcosine (Sar), α-aminoadipic acid (α-AAA), β-alanine (β-Ala), β-aminoisobutyric acid (β-AiBA), γ-aminobutyric acid (GABA), ethanolamine (EtONH$_2$), ammonia (NH$_3$), δ-allo-hydroxylysine (δ-HyLys), ornithine (Orn), 3-methylhistidine (MeHis), anserine (Ans), and carnosine (Car). The significance of overall concentration differences of the chemicals between male and female urine was tested using Wilcoxon matched pairs test.

Results

Ablation behavioral experiments

To identify the sensory organs of male helmet crabs involved in the detection of female sex pheromones, behavioral experiments were performed on selectively ablated crabs (Fig. 2). When the second antenna were ablated, 7 of the 9 male crabs tested showed grasping behavior, while 2 of them also showed copulation behavior toward sponges treated with postmolt female pheromone. With bilateral ablation of the inner flagella of the antennules, all 10 male crabs tested showed grasping behavior, and 5 of them also showed copulation behavior. In contrast, neither grasping nor copulation behaviors were observed in any of the 10 animals that had their antennular outer flagella bilaterally ablated. Differences in grasping or copulation behavior were not significant for crabs with their second antenna ablated and crabs with antennular inner flagella ablated

Figure 2. Behavioral responses of males with different sensory ablations to postmolt female aquarium water. **$P < 0.01$; *$P < 0.05$.

(Fisher's exact test, $P = 0.21$ and 0.35 respectively). The occurrence of both grasping and copulation behaviors of the crabs with outer flagella ablated was significantly different (Fisher's exact test, $P < 0.01$ and <0.05 respectively) from those of the crabs with inner flagella ablated. Between controls and crabs with outer flagella ablation, the difference in grasping behavior was significant (Fisher's exact test, $P < 0.01$), while it was not significant for copulation behavior (Fisher's exact test, $P = 0.47$).

Electrophysiological experiments

Since behavioral experiments indicated that the outer flagella of males are involved in perception of sex pheromones released from females, responses of chemosensory neurons in the outer flagellum were analyzed by extracellular multiunit recordings. (Chemoreceptors on the inner flagellum also responded to shrimp extract and male and female urines [pers. obs.], but they were not considered further here in our experiments.) In a typical recording, substantial spontaneous activity was observed throughout the experiment, probably reflecting the activity of tonically active mechanosensory afferents. Presentation of shrimp extract produced a phasic discharge of action potentials; this response occurred about 4 s after stimulus introduction, which reflects the time for the stimulus to reach the recording chamber and sensilla (Fig. 3A). During a response to chemical stimulation, several units with different spike amplitudes were active. However, the high spike frequency prevented reliable discrimination of single units (Fig. 3B, C). Therefore, the magnitude of the responses was evaluated by integration (top trace in Fig. 3A).

The activity of these chemosensory neurons did not change significantly when ASW was injected as a control (Fig. 4A). In contrast, the afferents were strongly activated after injection of female (Fig. 4C) or male urine (Fig. 4D), as well as after injection of shrimp extract (Fig. 4B). The relative magnitude of the response to female urine compared to the response to shrimp extract varied from 53% to 324% (148 ± 24; mean ± SEM, $n = 11$). The relative magnitude of the response to male urine compared to the response to shrimp extract ranged from 41% to 264% (127 ± 21; $n = 11$). The relative magnitude of the response to female urine compared to the response to male urine ranged from 99% to 147% (119 ± 5; $n = 11$), and this difference was statistically significant (Wilcoxon matched pairs test, $z = 2.84$, $P < 0.01$). After the ablation of the outer flagellum, the baseline amplitude of integrated activity declined due to a decrease in spike number of mechanosensory afferents innervating exteroceptors on the outer flagellum. Chemosensory responses were no longer observed after injection of shrimp extract (Fig. 4E).

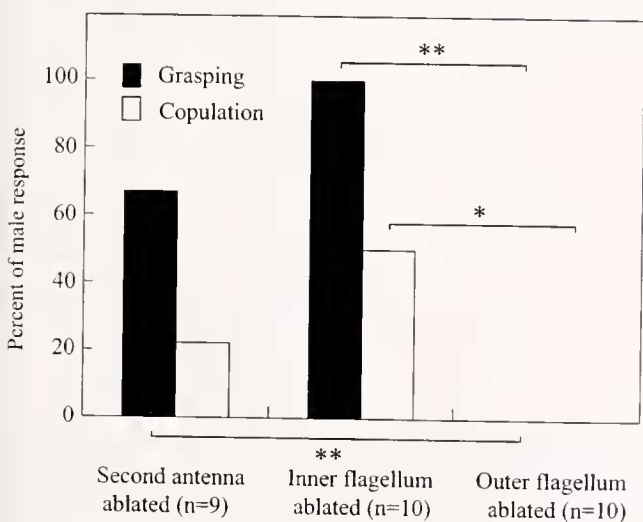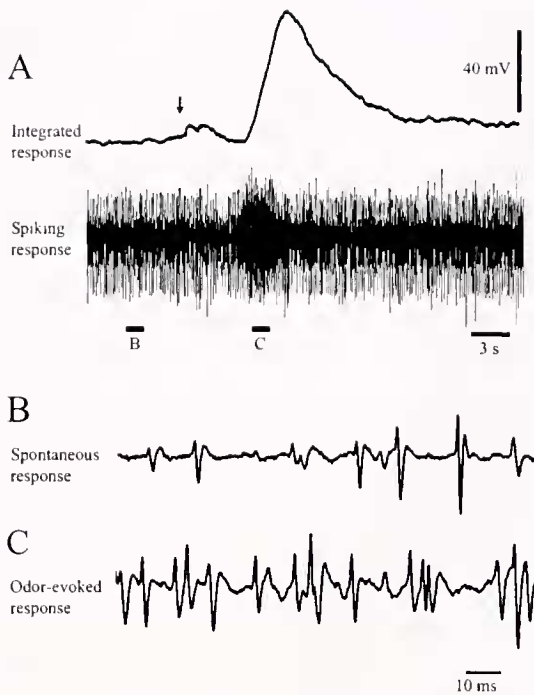To characterize the difference in the sensitivity of the

Figure 3. Responses of chemosensory neurons in the antennular outer flagellum of a male specimen of *Telmessus cheiragonus* to shrimp extract. (A) Extracellular recording from antennular nerve to the injection of shrimp extract (lower trace) and its integrated activity (upper trace). Time of injection is indicated by arrow. (B) Spike activity of the antennular nerve before injection of shrimp extract. (C) Spike activity of the antennular nerve in response to injection of shrimp extract. (B) and (C) correspond to part "B" and "C" of the lower trace of Figure 3A.

chemoreceptors on the outer flagellum to both female and male urines, the responses of outer flagellum afferents to various concentrations of female and male urine were analyzed quantitatively by normalizing them to the response to 1 mM L-serine (standard reference stimulus: STD) (Fig. 5). The outer flagellum showed larger responses to female urine than to male urine at all dilutions, and the differences were significant at dilutions of 10^{-3} ($z = 2.20$, $P < 0.05$: Wilcoxon matched pairs test) and 10^{-4} ($z = 1.99$, $P < 0.05$). Overall differences among ASW, female urine, and male urine were also significant (Friedman ANOVA, $P < 0.001$).

Amino acid analysis

Male and female urines contained detectable amounts of common amino acids and nitrogenous compounds; taurine, urea, and ammonia were most abundant in the urine, ranging from 49 to 248 μM, while the rest of the compounds were present at less than 20 μM. Concentrations of compounds were higher in male urine than in female urine, except for ethanolamine, γ-aminobutyric acid, and phos-

phoserine (Fig. 6). Overall, concentration differences of the chemicals between male and female urines were significant (Wilcoxon matched pairs test, $z = 4.52$, $P < 0.001$).

Discussion

Our ablation experiments suggest that the antennular outer flagellum of males is the site of reception of female

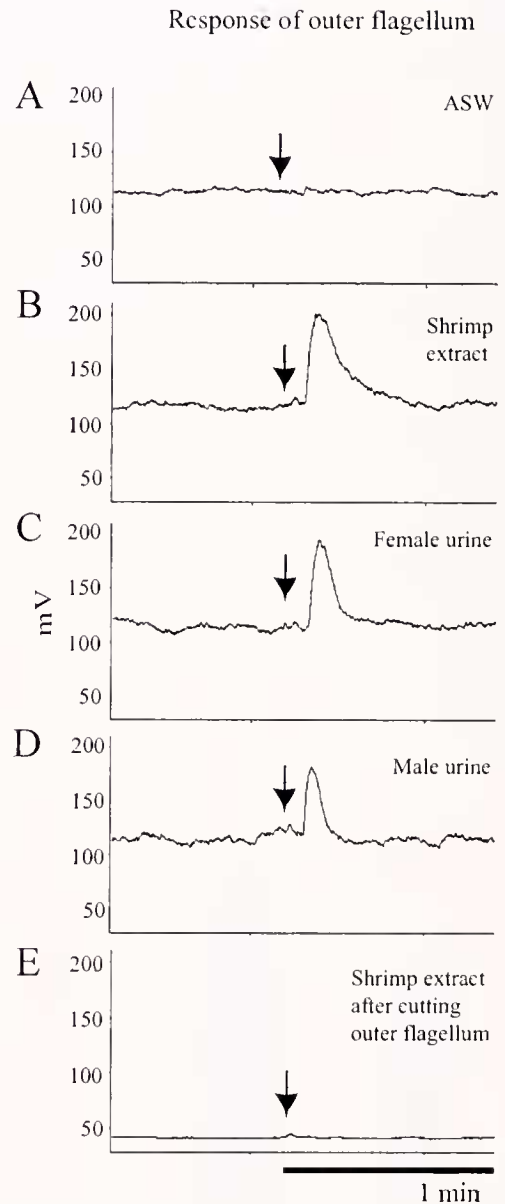### Response of outer flagellum

Figure 4. Responses of chemosensory neurons of the outer flagellum of a male specimen of *Telmessus cheiragonus* to various chemical stimuli. Integrated activity in response to artificial seawater (ASW) (A), shrimp extract (B), female urine (C), and male urine (D). No significant response to the injection of shrimp extract was observed after ablation of the outer flagella (E). Time of injection is indicated by arrow.
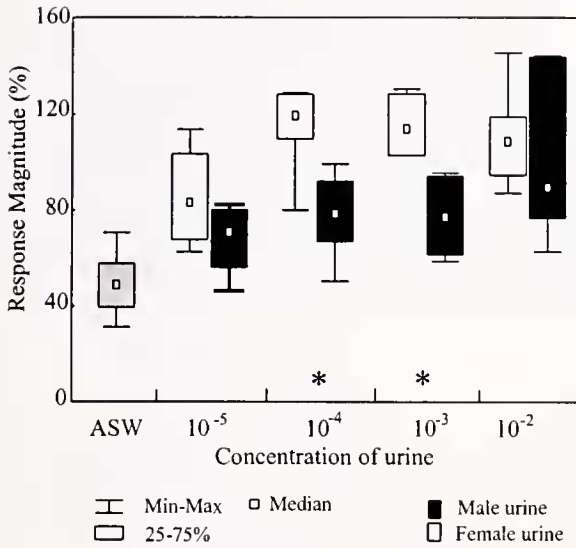
Figure 5. Concentration-response relationships of chemosensory afferents of the antennular outer flagellum of males of *Telmessus cheiragonus* to male and female urines. The response magnitudes were normalized to a percentage of the response to 10^{-3} *M* L-serine for each recording. The responses shown are the median, the 25%–75% quartiles, and the minimum-maximum values. Data are from 6 preparations. * indicates responses that are statistically different between male and female urine (Wilcoxon matched pairs test, $P < 0.05$).

pheromones in *Telmessus cheiragonus*. Furthermore, this behavioral observation is supported by our electrophysiological finding that chemosensory afferents of the outer flagellum respond more sensitively to female urine than to male urine, the former of which contained the sex pheromone that elicits grasping behavior (Kamio *et al.*, 2000). Thus, as with *Portunus sanguinolentus* (Christofferson, 1972) and *Callinectes sapidus* (Gleeson, 1982), *T. cheiragonus* detects sex pheromones by means of the antennular outer flagellum.

Urine of crustacean species contains many small nitrogenous molecules such as ammonia, urea, uric acid, and amino acids, which are nitrogenous excretory metabolites from tissues and hemolymph (Claybrook, 1983). Some of these nitrogenous compounds stimulate aesthetasc sensilla (Spencer, 1986; Derby and Atema, 1988). In the present study, many kinds of amino acids and nitrogen excretory products are found both in male urine and in female urine. The concentration of most of these compounds is higher in male urine than in female urine, and overall the amino acid concentration was higher in male urine than in female urine. Perhaps higher concentrations of these compounds in male
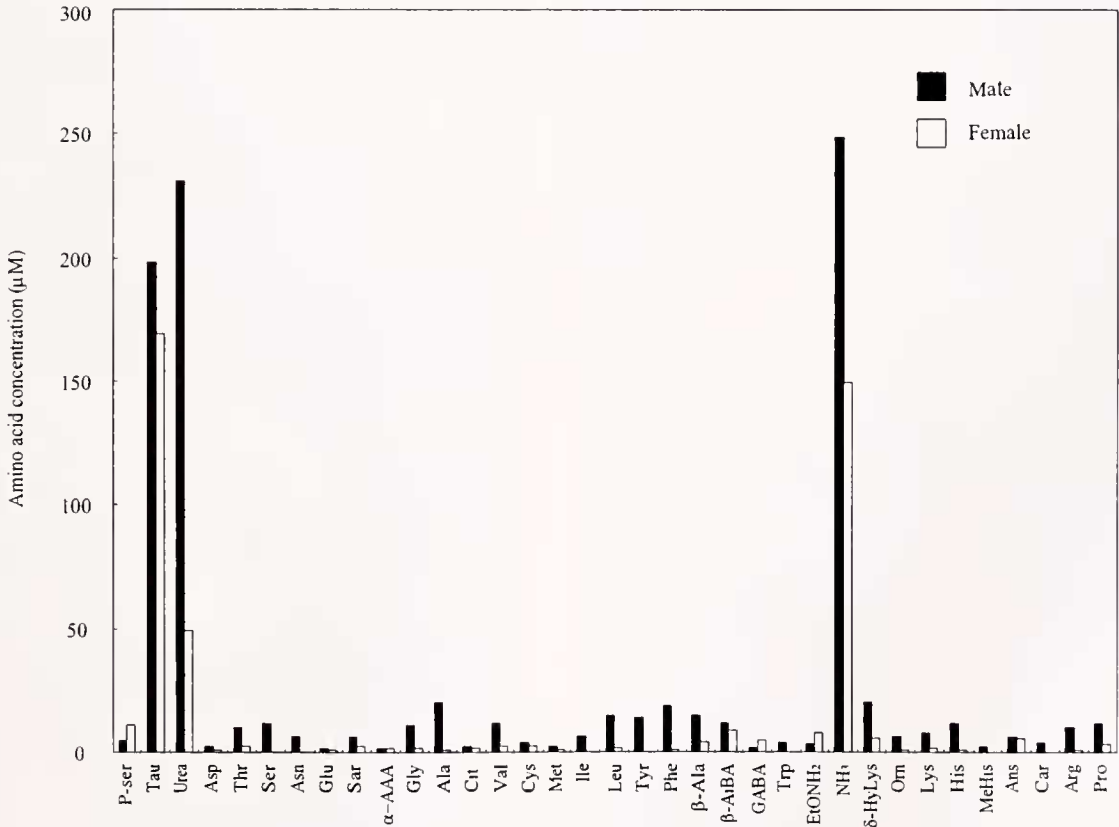

Figure 6. Concentrations of amino acids in urine of female and male specimens of *Telmessus cheiragonus*.

urine reflects the crabs' feeding history: premolt females do not eat and postmolt females were not fed during the period of urine collection, while males were fed shrimp. If the antennular chemosensory neurons respond sensitively to the nitrogenous compounds that have been detected in this amino acid analysis, the higher concentrations of nitrogenous compounds in male urine might be expected to cause a higher response of chemosensory afferents of the outer flagellum to male urine than to female urine (Fig. 4). However, the outer flagellum afferents showed significantly larger response to female urine at concentrations of less than 10^{-3} M (Fig. 5). The results demonstrate that compounds with higher abundance in male urine are not responsible for excitation of the afferents in the outer flagellum of male helmet crab. Compounds such as ethanolamine, GABA, and phosphoserine, which are more abundant in female urine than in male urine, may contribute to the excitation (Fig. 6). It is also possible that compounds not detected in the amino acid analysis contribute to the afferent response. Without the purified pheromone molecules, we can infer but not be certain that this differential electrophysiological responsiveness is due to sex pheromones.

At the moment, it still remains to be clarified exactly where the pheromone-specific receptors are located on the outer flagellum. Gleeson (1982) noted that the aesthetasc tuft on the outer flagellum of the antennule in *C. sapidus* is divided into mesial and lateral halves by a region of cuticle from which no sensilla arise. Ablation of mesial or lateral halves of the outer flagellum partially reduced the behavioral response of males to female sex pheromone, thus suggesting that pheromone receptors are located on both sides of the outer flagellum. Further anatomical and physiological studies will be necessary to clarify which sensilla on the outer flagellum of male helmet crab are responsible for detecting female pheromones.

Postmolt female urine contains grasping pheromone but not copulation-eliciting pheromone (Kamio *et al.*, 2002). Ablation of the outer flagellum eliminated both copulatory behavior and grasping behavior by male crabs toward postmolt-female-conditioned water. Although the present electrophysiological studies did not test the copulation pheromone, its reception site may also be on the outer flagellum. However, it is also possible that the reception site of copulation pheromone is not located on the antennular flagella. In this case, grasping pheromone may be necessary to elicit copulatory behavior. The release site of the copulation pheromone must be identified to answer this question.

Future studies will focus on identifying the sex pheromone molecules of the helmet crab. A combination of behavioral and electrophysiological techniques, such as used in this study, together with analytical chemistry, will guide the search.

Acknowledgments

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